Kinetic Study of the Jaffé Reaction for Quantifying Creatinine in Serum: 1. Alkalinity Controlled with NaOH

Harry L. Pardue, Bonnie L. Bacon, Marilyn Groeger Nevlus, and John W. Skoug

We studied the kinetic behavior of the reaction of alkaline picrate and creatinine and evaluated a nonlinear curve-fitting method for quantifying creatinine in serum. Using a $3 \times 3$ factorial experimental design, we evaluated interactive effects among temperature and concentrations of creatinine, picrate, and NaOH. We found no evidence of interference by glucose or unconjugated bilirubin; the effects of the acetoacetate reaction, which is fast, are easily compensated by the curve-fitting method. The reaction with human serum albumin is very complex, but its effects are compensated by the curve-fitting method and by preparing standards containing 50 g of albumin per liter. Calibration plots are linear under a wide variety of conditions for both aqueous standards and standard additions of creatinine to pooled serum. Reproducibility studies with standards containing creatinine at 2, 10, and 20 mg/L yielded relative standard deviations (RSD) of 8.2, 2.5, and 1.3%, corresponding to absolute variations of 0.16, 0.25, and 0.26 mg/L. The average SD for 17 sera containing creatinine at 15–50 mg/L was 0.7 mg/L. The averages of ratios (as percent) of determined vs expected concentrations in 17 sera with added creatinine (7.27 mg/L) were 97.8% for aqueous standards, 99.9% for standards with added albumin.

Since the early work of Folin (1), many have attempted to improve performance characteristics of the Jaffé reaction for the quantification of creatinine in body fluids. More recent studies have emphasized the use of the kinetic phase of the reaction to reduce effects of interferences. Recent reviews (2, 3) have discussed principles and practical developments in this area. Although substantial improvements have been made, these reviews make it clear that significant problems and uncertainties regarding effects of interferences remain. Despite the trend toward the use of enzymes to improve selectivity for creatinine, the Jaffé reaction still is used extensively, and a more nearly complete understanding of this reaction system is needed than exists now.

Vasiliades (4) has reported a rather detailed study of the kinetics and mechanism of the alkaline-picrate/creatine reaction. However, conditions used in that study were selected to optimize kinetic/mechanistic information obtained rather than practical applications of the reaction. Other studies have focused more attention on the performance characteristics of preselected reaction conditions (3) than on the effects of different reaction variables. Others have examined reaction variables, but with primary focus on equilibrium-based approaches (5).

Our objectives here were to study in detail the kinetic behavior of the reaction, including the effects of the reaction variables, the effects of interferents on the reaction, the impact of information obtained on the quantitative procedures for creatinine, and the potential utility of a multipoint curve-fitting method (6, 7) for the kinetic quantification of creatinine in serum.

Our focus in this study was on unbuffered solutions, with sodium hydroxide used to adjust alkalinity. We examined the effects of temperature and concentrations of picrate, sodium hydroxide, and creatinine on the sensitivity and pseudo-first-order rate constants. We also evaluated the behavior of selected interferents (glucose, acetoacetate, human serum albumin, and "unconjugated" bilirubin) for one set of reaction conditions. We report variable dependencies, both in the classical two-dimensional format and as pseudo-three-dimensional plots that aid visualization of the interactive effects of different variables. We also present quantitative results for creatinine in aqueous standards and sera.

Materials and Methods

Instrumentation

Data for absorbance vs time were obtained with a diode-array-based spectrophotometer (Model 8450; Hewlett-Packard, Palo Alto, CA) and a centrifugal mixing system (Rotochem IIIA; Aminco, Deerfield, IL). Reagents and samples were mixed manually in the diode-array-based spectrophotometer and added manually to the rotor of the centrifugal mixer. In each, temperature was controlled by water circulated from a constant-temperature water bath.

Data were collected by on-board computers in each instrument and transferred to a supermicro processor (Masscomp 510 work station; Masscomp, Westford, MA), in which is used a "unix" operating system; programs were written in "C language" to be easily transferable to other similar systems (8).

Reagents

All solutions were prepared in distilled de-ionized water.

**Alkaline picrate.** Working alkaline picrate reagents were prepared by diluting stock solutions that contained 6 mol of NaOH (Mallinckrodt Inc., Paris, KY) and 28.5 mmol of picric acid (MCB Reagents, EM Science, Gibbstown, NJ) per liter. Working reagents were refrigerated in amber-colored glassware.

**Creatinine.** A stock 1.00 g/L creatinine (Sigma Chemical Co., St. Louis, MO) solution was prepared in 0.1 mol/L hydrochloric acid. Working standards were prepared by diluting this stock solution.

**Other.** A stock 2.1 g/L solution of glucose (Pfanstiehl Laboratories, Waukegan, IL) and a 100 g/L solution of human serum albumin (HSA, Cohn Fraction V, cat. no. A1653; Sigma) were prepared in water.

Acetoacetate stock solution, 236 mmol/L, was prepared by distilling ethyl acetoacetate (Matheson, Coleman, and Bell, Norwood, OH), diluting a 0.75-mL aliquot of the distillate (b.p. fraction 176–177 °C) to 25 mL with 0.2 mol/L sodium hydroxide, and allowing this to hydrolyze for 48 h at 4 °C.

A 300 mg/L stock standard of bilirubin (unconjugated, cat. B4126, Sigma) was prepared by dissolving 30.0 mg of bilirubin in 1.0 mL of dimethyl sulfoxide and 3.0 mL of
sodium carbonate (0.1 mol/L), then diluting to 100 mL. Another solution of bilirubin at 20 mg/L was prepared by diluting an aliquot of this stock; working solutions were prepared by diluting stock solutions twofold with creatinine (0.0–60.0 mg/L) solutions.

Procedures

**Mixing, measurement steps.** We incubated reagents, standards, and samples in a water bath at the desired temperature for 15 min before initiating the reactions. For all studies, we used equal volumes of the alkaline picrate reagent and standards or samples. For studies with the diode-array-based spectrophotometer, reagent and sample were mixed externally, then quickly placed in the cuvette. For the centrifugal mixer, equal volumes (250 μL) of reagent and sample were added to the inner and outer rotor wells, respectively. We measured absorbance at 2-s intervals for 800 s—at 490 nm with the recording spectrophotometer, at 520 nm with the centrifugal mixing system.

**Response-surface experiments.** We used response-surface methodology (9, 10) to study the effects of sodium hydroxide, picrate, and temperature on the first-order rate constant and sensitivity. Table 1 lists the values for each factor used in a full factorial experimental design. We included two replicates at selected factor combinations to assess the goodness-of-fit of the quadratic regression model to each of the experimental response surfaces studied. For each set of factor combinations, we analyzed seven creatinine standards with concentrations between 2 and 30 mg/L (before mixing). The experiments were randomized and performed over a three-day period. Working solutions of the creatinine standards were prepared on the first day and refrigerated until needed. The Jaffé reagent was freshly prepared each day.

**Data processing.** Two levels of data processing were required. First, the raw data for absorbance vs time were processed with a first-order model. The nonlinear least-squares regression program (6, 7) was used to compute the pseudo-first-order rate constant, $k_{\text{app}}$, and the absorbance change, $\Delta A_{s}$. Second, the average of the rate constants over all creatinine concentrations, and the least-squares slopes (sensitivities) of the absorbance change vs creatinine concentration for each combination of factors, were used as data for a program to compute response surfaces. The program computes the least-squares parameters that provide the best fit of the experimental data to a quadratic model. We also used statistical tests to assess the goodness-of-fit of the regression model, the significance of the individual factors, and the relative importance of the linear, quadratic, and interaction terms in the model. Results are reported in the classical two-dimensional format to aid in the visualization of individual dependencies and as pseudo-three-dimensional plots to help identify interactions among the variables.

**Results and Discussion**

Unless stated otherwise, imprecision is reported as one standard deviation (±1 SD).

**Response Curves**

Figure 1 shows response curves for three concentrations of creatinine. The points represent experimental data and the smooth curves represent fits of data, between 10 and 80 s and 10 and 140 s, to a first-order model. The shorter fitting range (about one half-life) does not give good agreement between experimental and computed values of absorbance, but the longer fitting range (about two half-lives) does, as do fitting ranges up to 800 s.

For good first-order behavior, the apparent rate constant should be independent (within experimental error) of creatinine concentration. Rate constants computed by applying the curve-fitting process to data from 10 to 400 s for 20 creatinine concentrations between 2 and 40 mg/L (under conditions as in Figure 1) gave an average value of (9.82 ± 0.45) × 10⁻⁴ s⁻¹ with a total range from 10.7 × 10⁻⁴ (at 2 mg/L) to 9.1 × 10⁻⁴ s⁻¹ (at 20 mg/L). Reproducibility studies with creatinine concentrations of 2, 10, and 20 mg/L (15 runs each, conditions as in Figure 1) yielded average values for $k$ of 11.6 ± 0.77, 10.2 ± 0.18, and 10.1 ± 0.19 × 10⁻⁴ s⁻¹, respectively. These and other results indicate a small systematic trend toward a decreasing rate constant with increasing creatinine concentrations. This result and the poor fit obtained with data between 10 and 80 s (Figure 1) indicate that the reaction does not follow exact first-order kinetics. However, for fitting ranges of two half-lives ($t_{1/2} = 69$ s) or more, the effects of deviations from first-order behavior are very small, and the first-order curve-fitting process should be satisfactory for evaluations of parameter dependencies and for quantifying creatinine.

Although linearity is discussed in detail later, we note here that the computed absorbance change, $\Delta A_{s}$, varies linearly with creatinine concentration, $C_{s}$, under a wide range of experimental conditions.

**Table 1. Experimental Design for Response-Surface Experiments**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature, °C</td>
<td>25.0, 32.0, 37.0</td>
</tr>
<tr>
<td>Picrate concn, mmol/L</td>
<td>1.0, 4.0, 8.0*</td>
</tr>
<tr>
<td>NaOH concn, mmol/L</td>
<td>50, 300, 600</td>
</tr>
</tbody>
</table>

* A full factorial design with two replicates (n = 29) was used. * Sodium hydroxide concentration was decreased to 550 mmol/L because of precipitation.

**Fig. 1.** Response curves for creatinine standards at (bottom to top) 2, 10, and 20 mg/L Experimental (Δ) and fitted data with fitting ranges of 10–80 s (---) and 10–140 s (→)
Parameter Dependencies

As noted in the previous section, the rate constant varies only slightly with creatinine concentration and absorbance change varies linearly with creatinine concentration. Accordingly, to simplify the presentation of the large amounts of data obtained, we report rate constants averaged over all values of creatinine concentration for each set of conditions. We report sensitivities: slopes of least-squares fits of absorbance change, ΔA/τ vs creatinine concentration, in units of liter per milligram. These sensitivities can be converted to apparent molar absorptivity, if 1 mol of product per mole of creatinine is assumed, by multiplying sensitivities by 1000 times the molecular mass of creatinine.

Combined dependencies. We used response-surface methodology not only to aid in graphic presentation of the results but also to predict the response under selected conditions not included in the experimental design. The experimental values of sensitivity and average rate constant for each of the 29 combinations of variables were fit to a quadratic model of the form:

\[ Y = b_0 + b_1 X_1 + b_2 X_2 + b_3 X_3 + b_{11} X_1^2 + b_{22} X_2^2 + b_{33} X_3^2 + b_{12} X_1 X_2 + b_{13} X_1 X_3 + b_{23} X_2 X_3 \]

where \( Y \) is the response being modeled (rate constant or sensitivity); \( X_1, X_2, \) and \( X_3 \) are the respective variables of temperature, picrate, and NaOH concentrations; and the \( b \)'s are coefficients that give the best fit of the model to the experimental data. The regression results were computed by sequentially adding the linear terms (i.e., terms with \( b_1 \) to \( b_3 \)), the quadratic terms (those with \( b_{11} \) to \( b_{33} \)), and finally the interaction terms into the regression equation. At each stage of this model-building process, an F-ratio is calculated based on the extra sum of squares due to regression \((II)\); this indicates the relative collective importance of each set of terms in explaining the error in the response surface.

Table 2 summarizes the analysis-of-variance results computed by this procedure. For each response studied, a large percentage of the variation in the response variable (78% and 88% for the rate constant and sensitivity, respectively) is explained by the linear terms in equation 1. This interpretation is consistent with visual observations made with more conventional two-dimensional plots.

It is useful to quantify some of these linear dependencies here. For NaOH concentration at 300 mmol/L and 32 °C, a linear least-squares fit of rate constant \( k \) (s⁻¹) vs picrate concentration, \( C_{JH} \) (mmol/L), yielded \( k = (1.9 \times 10^{-5}) C_{JH} \) + (5.9 \( \times \) 10⁻⁴) s⁻¹. For picrate concentration at 4 mmol/L and 32 °C, a linear least-squares fit of rate constant vs NaOH concentration, \( C_{NaOH} \) (mmol/L), yielded \( k = (3.1 \times 10^{-5}) C_{NaOH} \) - (6.3 \( \times \) 10⁻⁴) mmol/L. For NaOH and picrate concentrations of 300 and 4 mmol/L, respectively, a linear least-squares fit of \( k \) vs \( T \) (°C) yielded \( k = (3.2 \times 10^{-4}) T \) - (2.1 \( \times \) 10⁻³) s⁻¹.

Although the sensitivity varies only slightly with picrate concentration, it varies approximately linearly with temperature. For NaOH and picrate concentrations of 300 and 4 mmol/L, respectively, the linear least-squares fit of sensitivity (L · mg⁻¹) vs temperature (°C) yielded \( \Delta A_{C,T} = (5.2 \times 10^{-4} T) + (8.8 \times 10^{-5}) L \cdot mg^{-1} \). It is generally recognized that rate constants increase with temperature \((2, 3)\); however, equilibrium constants and absorptivities usually depend much less on temperature than do rate constants. However, as shown in Figure 2, not only the rate but also the total absorbance increases with increasing temperature, an observation consistent with earlier studies \((II)\). Practical consequences of this are that both equilibrium and kinetic methods for creatinine require careful control of temperature; moreover, the proposed curve-fitting kinetic method will not be as insensitive to variations in temperature, as is the method for other reactions \((6, 7)\).

Table 2 also shows the presence of strong interactive effects, but few curvature effects, over the range of variables studied. Figure 3 illustrates one such interactive effect, of NaOH concentration on sensitivity at different temperatures. Although the dependence is approximately linear at 25 °C, increasing degrees of curvature are apparent at higher temperatures, as well as a decrease in sensitivity at

\[
\begin{array}{|c|c|c|c|}
\hline
\text{Terms added} & r^2 & F\text{-ratio} & F\text{-ratio} \\
\hline
\text{Linear} & 0.785 & 642.7 & 0.877 & 214.7 \\
\text{Quadratic} & 0.004 & 3.4 & 0.013 & 3.1 \\
\text{Interaction} & 0.203 & 166.1 & 0.065 & 20.7 \\
\hline
\text{Total} & 0.992 & 270.7 & 0.974 & 79.5 \\
\hline
\end{array}
\]

The F-ratio is used to test the null hypothesis that all coefficients in a group are zero.

![Fig. 2. Effects of temperature (bottom to top: 28, 30, 32, and 35 °C) on response curves](image)

![Fig. 3. Effects of NaOH concentration on sensitivity](image)
low NaOH concentration and 37 °C. We are at present unable to explain this behavior. Equation 1 presents a more detailed view of all the dependencies.

Table 3 lists least-squares estimates of the coefficients of equation 1, with special attention to the coefficients that differ significantly from zero. However, because of the inherent multicollinearity among the independent variables of the quadratic model (12), the coefficients cannot be used to assess the effect of each factor independently of the other two. Nonetheless, this does not degrade the usefulness of the regression results, because excellent fits were obtained for both responses studied. Accordingly, we used the coefficients listed in Table 3 to predict responses for values of the variables not included in the experimental study (but within the limits of Table 1) and to compute the response surface in Figure 4. We illustrate response surfaces for only one temperature (32 °C) because the slopes of other surfaces are similar, differing primarily in amplitude. These response surfaces provide the best estimates of the responses expected for variable combinations not included in the experimental study, and can be used to identify conditions that might be selected to accomplish specific objectives. For example, to optimize sensitivity, one would use a low concentration of NaOH, a high concentration of picrate, and a high temperature (see Figure 4, top). Or, to minimize measurement time by increasing the reaction rate, one would select high values of all three variables considered here (Figure 4, bottom).

For practical applications, it is necessary to make a compromise between these options. Unless stated otherwise, for the remainder of this study we chose picrate and NaOH concentrations near 6 and 300 mmol/L, respectively; a temperature of 30 or 32 °C as reasonable compromises between speed and sensitivity; and a fitting range of 10–140 s. A higher temperature and a lower NaOH concentration would probably permit similar measurement times, but with improved sensitivity.

Reproducibility and Linearity

To evaluate reproducibility, we did 15 experiments each on three aqueous solutions of creatinine at concentrations of 2, 10, and 20 mg/L. Average absorbance changes were 0.0772, 0.3797, and 0.7528, respectively, with SDs of 0.0059, 0.0094, and 0.0096, corresponding to relative standard deviations (CVs) of 9.2, 2.5, and 1.3%. The pooled SD for these data was 0.0084 (RSD = 2.1%).

As noted earlier, computed values of ΔA∞ varied linearly with creatinine concentration under a wide variety of conditions. For 20 equally spaced creatinine concentrations (2–40 mg/L) with a 10- to 140-s fitting range, the linear least-squares statistics are

\[ \Delta A_\infty = (0.0395 \pm 0.00046) C_{CL}^0 - (0.015 \pm 0.01), \]

a standard error of estimate (s_y) = 0.024 L·mg⁻¹, and correlation coefficient (r) = 0.998. Uncertainties are approximately halved when the concentration range is limited to 2–20 mg/L. When the fitting range was extended to 800 s (11.4 half-lives), where the reaction has approached completion, the least-squares statistics for this same data set were \[ \Delta A_\infty = (0.0390 \pm 0.00039) C_{CL}^0 - (0.0027 \pm 0.0009), \] s_y = 0.02 L·mg⁻¹, and r = 0.998. There is an apparent improvement in the intercept, but the uncertainty in the intercept is essentially the same for the two fitting ranges.

Interferences

The most serious problem associated with the picrate method for creatinine is that of interferences. Bowers and Wong (3), discussing this problem in detail, noted inconsis-

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**Fig. 4. Effects of picrate and NaOH concentrations on sensitivity and rate at 30 °C**

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**Table 3. Regression Results from Fitting Values for Rate Constants and Sensitivity to Equation 1**

<table>
<thead>
<tr>
<th>Coefficient</th>
<th>Factor</th>
<th>( k_{obs} (10^{-3} \text{ s}^{-1}) )</th>
<th>Sensitivity (10⁻³ L/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>b₀</td>
<td>Intercept</td>
<td>15.677</td>
<td>21.700</td>
</tr>
<tr>
<td>b₁</td>
<td>Temp.</td>
<td>-0.672</td>
<td>-0.019</td>
</tr>
<tr>
<td>b₂</td>
<td>Picrate</td>
<td>-2.065*</td>
<td>2.630*</td>
</tr>
<tr>
<td>b₃</td>
<td>NaOH</td>
<td>-0.040*</td>
<td>-0.026*</td>
</tr>
<tr>
<td>b₁₁</td>
<td>Temp. + temp.</td>
<td>0.004*</td>
<td>-0.001*</td>
</tr>
<tr>
<td>b₂₂</td>
<td>Picrate + picrate</td>
<td>-0.063*</td>
<td>-0.080*</td>
</tr>
<tr>
<td>b₃₃</td>
<td>NaOH + NaOH</td>
<td>8.846 \times 10⁻⁶</td>
<td>-3.27 \times 10⁻⁶</td>
</tr>
<tr>
<td>b₁₂</td>
<td>Temp. + NaOH</td>
<td>0.082*</td>
<td>0.027*</td>
</tr>
<tr>
<td>b₁₃</td>
<td>Picrate + NaOH</td>
<td>0.001*</td>
<td>0.001*</td>
</tr>
<tr>
<td>b₂₃</td>
<td>Picrate + NaOH</td>
<td>0.007*</td>
<td>-0.004*</td>
</tr>
</tbody>
</table>

*Significantly different from zero at the 95% confidence limit.
tencies in the literature regarding effects of some interferents. We chose to examine the effects of four compounds—glucose, acetoacetate, human serum albumin, and bilirubin—under some of the reaction conditions studied here. We used both fast (stopped-flow) and slow mixing methods to study the effects of glucose, bilirubin, and acetoacetate. Results for all four compounds are summarized in Figure 5 and discussed below.

Glucose. For glucose, we observed no measurable reaction during the early part (10 ms to 5 s) of the reaction. However, there appears to be a very gradual increase in absorbance with time for the 1050 mg/L glucose sample alone with the picrate reagent, and the response curve for a mixture of creatinine and glucose is slightly above that for creatinine (Figure 5A), although the difference is close to the experimental uncertainty in the data. This is consistent with earlier observations (13). Subtracting the data for glucose alone from those for the mixture of glucose and creatinine gives a slight negative slope to the difference curve at longer times, indicating that the mixture curve does not include the gradually increasing component observed for glucose alone. Further to evaluate the effects of glucose, we did experiments in which seven creatinine concentrations (0–30 mg/L) were quantified in the presence and absence of glucose, and computed linear least-squares statistics of ΔΔA values obtained in the two situations. The slope and intercept (ΔΔA for mixture vs ΔΔA for creatinine alone) were 0.999 ± 0.008 and 0.004 ± 0.003 mg/L, respectively. The slope and intercept are not significantly different from ideal values of unity and zero. We conclude that if there is any effect of glucose for these conditions, it is quite small and close to the experimental error.

Bilirubin. Our data (Figure 5B) show no fast or slow time-dependent reaction of bilirubin with the picrate reagent. The only apparent effect of bilirubin is to increase the blank absorbance, which is automatically compensated with the curve-fitting and other kinetic methods. A linearity study for creatinine with bilirubin (150 mg/L) and without bilirubin (see description for glucose) yielded a least-squares slope and intercept of 1.04 ± 0.01 and 0.0044 ± 0.004, respectively. For bilirubin at 10 mg/L, slope and intercept were 1.02 and 0.002, respectively. We conclude that the form of bilirubin used in this study offers little or no interference; other forms may react differently.

Acetoacetate. Stopped-flow data showed a smooth, near first-order, reaction of acetoacetate with picrate (Figure 5C). This is consistent with earlier observations (12, 18). The reaction is complete within a few seconds and behaves essentially as a sample blank at times longer than 20 s. This is reflected in the similarity of curves b and e in Figure 5C. Linearity studies similar to those described for glucose yielded a slope and intercept of 0.978 ± 0.01 and 0.008 ± 0.0044, respectively, showing that the curve-fitting method compensates effectively for the acetoacetate reaction.

Human serum albumin. Results for human serum albumin are more surprising and complex (Figure 5D). During
the first 400 s, there is a gradual, apparently linear, increase in absorbance with time, after which the rate of absorbance change accelerates rapidly and continues to increase throughout the 800-s monitoring time. We had previously observed this type behavior with sera (see Figure 6), with the acceleration in rate always occurring near 400 s for all serum samples examined, but had been unable to explain it until we obtained these results for human serum albumin. This behavior was not noted in an earlier study, but the reaction was monitored only for 130 s (23).

The general behavior is the same with or without creatinine, but the quantitative behavior is not independent of creatinine concentration. For the particular human serum albumin concentration used in this study, at both low (4 mg/L) and high (30 mg/L) creatinine concentrations, the rate of acceleration after 400 s is faster in the absence than in the presence of creatinine. Also, human serum albumin appears to alter the characteristics of response curves during the early part of the reaction, but the curve-fitting method can still be used to compute an absorbance change, analogous to that computed in the absence of human serum albumin. Furthermore, ΔA values computed in this way vary linearly with creatinine concentration. However, the slope of the calibration curve in the presence of human serum albumin (50 g/L) is less for creatinine alone (0.0232 ± 0.00017 vs 0.0253 ± 0.00017) and the intercept is larger in the presence than in the absence of human serum albumin (0.054 vs −0.001).

Serum Samples

Response curves. Figure 6 shows response curves for absorbance vs time for 10 repetitive runs on a pooled serum sample as well as a representative fit of the data from 10 thru 140 s to a first-order model (dashed curve). The general shapes of the response curves, which were the same for all sera examined, are the same as those for human serum albumin reacting with alkaline picrate (Figure 5D). Albumin (and perhaps other proteins) in serum is causing the acceleration of the absorbance change at longer times. These and other results show that the process is reproducible for a particular set of conditions. Finally, the fitted curves are all similar to the one shown, demonstrating that the fitting process is reproducible and that it can project absorbance values at infinite time that might be measured in the absence of the albumin reaction.

Standard addition to pooled sera. Two types of standard-addition (analytical-recovery) studies were done, one in which known amounts of creatinine (0 through 6 mg/L) were added to aliquots of pooled sample and another in which a known fixed amount of creatinine was added to different individual serum samples.

We did the first type of experiment at 32°C with five different combinations of picrate and NaOH concentrations (mmol/L) as follows (picrate, NaOH): 6, 300; 10, 300; 6, 500; 6, 100; and 2, 300. We processed kinetic (A vs t) data for aqueous solutions (used for standardization) and sera by an initial-rate method (least-squares slopes of dA/dt of seven data points from 10 to 30 s), a two-point, fixed-time method (ΔA, between 10 and 120 s), and the curve-fitting method (ΔA based on fits from 10 to 140 s). Creatinine concentrations in sera with standard additions were computed both directly (from values of dA/dt, ΔA, and ΔA) and indirectly (as the sum of the creatinine concentration in the pooled serum with no creatinine added plus the amount (concentration) of creatinine added for each standard-addition sample).

We then processed the determined (Cp) and expected (Cp) values in two ways, namely by computing least-squares slopes and intercepts of plots of Cp vs Cg and the ratios, 100 Cp/Cg (as percent). Results are summarized in Table 4.

If there were no complicating factors, plots of Cp vs Cg should be linear, with intercepts equal to the creatinine concentration in the serum pool and slopes equal to unity. Also, the ratios, 100 • (Cp/Cg), analogous to "recovery," should be 100% and the rate constants should be the same for sera and standards for each set of conditions. For all conditions and all data-processing options, plots of Cp vs Cg were linear as expected. For all plots except those for the lowest concentrations of picrate (2 mmol/L) and NaOH (100 mmol/L), standard errors of estimate (s) were all < 0.2 mg/L and correlation coefficients were ≥ 0.99. The most easily interpreted results are the slopes and the ratios of determined to expected concentrations. For the initial-rate and two-point methods, all slopes are less than unity; for the curve-fitting method, only the slopes for the very low

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**Figure 6.** Response curves for pooled serum: —, response curves (10 repetitions); - - - - , data between 10 and 140 s for one sample fitted to first-order model.

**Table 4.** Results for Standard Additions of Creatinine to Pooled Serum

<table>
<thead>
<tr>
<th>Reagent concn, mmol/L</th>
<th>Initial-rate</th>
<th>Two-point</th>
<th>Curve fitting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Picrate: NaOH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6:300</td>
<td>0.86</td>
<td>9.36</td>
<td>98(2.9)</td>
</tr>
<tr>
<td>10:300</td>
<td>0.86</td>
<td>9.35</td>
<td>97(2.8)</td>
</tr>
<tr>
<td>6:500</td>
<td>0.84</td>
<td>9.28</td>
<td>94(2.0)</td>
</tr>
<tr>
<td>6:100</td>
<td>0.65</td>
<td>6.20</td>
<td>92(6.8)</td>
</tr>
<tr>
<td>6:300</td>
<td>0.73</td>
<td>9.89</td>
<td>101(5.0)</td>
</tr>
</tbody>
</table>

* Slope of determined vs added concentration (0-6 mg/L). * Intercept, mg/L. * Averages (and SD) of n = 7 ratios of determined (Cp) vs expected (Cp) concentration as % (100 Cp/Cg).
concentrations of NaOH or picrate are significantly less than unity. Although most of the ratios of determined vs expected concentrations are close to 100%, attention should be paid to the standard deviations (in parentheses). In all but one case, standard deviations decrease in going from the initial-rate to the two-point to the curve-fitting method. In all cases, the concentration ratios tend to decrease as the amount of creatinine increases, but the range is much less for the curve-fitting method than for the other methods, as indicated by the standard deviations. Slopes, concentration ratios, and standard deviations are closer to desired values for the curve-fitting method than for either of the other, and the two-point method is superior to the initial-rate method.

The intercepts should represent creatinine concentration in the pooled serum and, ideally, all values should be the same. Although these results are difficult to interpret because we do not know the true value of creatinine concentration in the pooled serum, some useful observations can be made. Standard deviations for intercepts range from 0.03 to 0.29 mg/L, with most being in the range of 0.06 mmol/L. Thus, differences observed are judged to be “statistically” significant. There is good agreement between the intercept values in Table 4 and corresponding values of creatinine concentration determined directly. The largest difference was 8.86 vs 9.69 mg/L for the initial-rate method for picrate at 2 mmol/L and NaOH at 300 mmol/L; other differences ranged from 0.01 to 0.3 mg/L. In general, there is better agreement between results by the two-point and curve-fitting methods, with the initial-rate results tending to be lower than by these options. Results for low picrate and NaOH concentrations indicate that the curve-fitting method is less dependent on reaction conditions than the initial-rate and two-point methods. We also noted that rate constants were significantly lower for sera than for aqueous standards.

These results indicate that conditions involving picrate and NaOH at 6 and 500 mmol/L, respectively, could be expected to yield slope and concentration ratio closer to expected values; however, the high viscosity of this reagent presents a problem. We chose picrate and NaOH concentrations of 6 and 300 mmol/L for subsequent studies. Also, because all slopes are less than unity, it is clear that some component in serum is affecting the sensitivity of the method relative to aqueous standards. Comparison of response curves for sera and for creatinine with albumin indicated that effects were similar. Therefore, in subsequent studies, we compared results with standards in both water and human serum albumin matrices.

**Standard addition—individual sera.** Several standard-addition (recovery) experiments were done for individual sera with the curve-fitting method. As noted above, we computed results with creatinine standards prepared both in water and in albumin solution; results are summarized in Table 5. In each case, there is reasonable agreement between “expected” and “determined” values for sera with creatinine added; however, values obtained on using standards with albumin agree more closely than those without it. Average values of concentrations determined with the two types of standards agree quite well. The average difference, without regard to sign, is 0.73 mg/L. The average of standard deviations for repeat runs for the several samples is 0.7 mg/L. Therefore, it is difficult to conclude with certainty that there is any significant difference among results for the two types of standards. However, with a few exceptions, results with standards containing human serum albumin tend to be 0.3 to 0.8 mg/L lower than results with aqueous standards. Because of the similarities in response curves, we tend to favor the use of standards with albumin added.

In conclusion: there is a wide variety of conditions that will yield linear relationships between absorbance change and creatinine concentration. Before one can select an "optimum" set of conditions one must define explicitly what features one wishes to optimize. We chose conditions that represent a compromise between sensitivity and required measurement time. We found no evidence of reaction between alkaline picrate and glucose or bilirubin, and effects

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**Table 5. Results for Standard Addition of Creatinine to 17 Sera**

<table>
<thead>
<tr>
<th>Aqueous standards</th>
<th>Standards containing HSA, 50 g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Concn, mg/L</strong></td>
<td><strong>Concn, mg/L</strong></td>
</tr>
<tr>
<td>Expected a</td>
<td>Deter.</td>
</tr>
<tr>
<td>19.6</td>
<td>19.2</td>
</tr>
<tr>
<td>17.8</td>
<td>16.8</td>
</tr>
<tr>
<td>34.3</td>
<td>33.0</td>
</tr>
<tr>
<td>16.4</td>
<td>15.8</td>
</tr>
<tr>
<td>14.5</td>
<td>14.5</td>
</tr>
<tr>
<td>18.1</td>
<td>17.9</td>
</tr>
<tr>
<td>18.5</td>
<td>18.0</td>
</tr>
<tr>
<td>16.3</td>
<td>15.6</td>
</tr>
<tr>
<td>19.6</td>
<td>18.4</td>
</tr>
<tr>
<td>49.5</td>
<td>49.2</td>
</tr>
<tr>
<td>30.0</td>
<td>28.6</td>
</tr>
<tr>
<td>17.8</td>
<td>17.7</td>
</tr>
<tr>
<td>18.5</td>
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<tr>
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<td>18.4</td>
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<tr>
<td>17.9</td>
<td>17.4</td>
</tr>
<tr>
<td>16.2</td>
<td>16.2</td>
</tr>
<tr>
<td>48.0</td>
<td>48.4</td>
</tr>
<tr>
<td>23.0</td>
<td>22.6</td>
</tr>
</tbody>
</table>

a: For undiluted serum + added creatinine, 7.27 mg/L. b: 100 CONC/CRE. c: Difference between determined values for standards with and without human serum albumin. d: Average of absolute values.
of reactions involving acetoacetate and albumin are compensated by the curve-fitting method. Data in Table 5 strongly indicate that serum matrices do not introduce significant proportional errors but give no information regarding possible additive errors. However, the fact that species such as glucose, acetoacetate, bilirubin, and albumin that are expected to interfere in some procedures do not interfere or are compensated with this method lends some indication that additive errors may be small.

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References